PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 11402-98	**FOR FURTHER ACTION** See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/CA 03/01323	International filing date *(day/month/year)* 22.08.2003	Priority date *(day/month/year)* 22.08.2002
International Patent Classification (IPC) or both national classification and IPC C07K14/47		
Applicant NATIONAL RESEARCH COUNCIL OF CANADA et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 12 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 9 sheets.

3. This report contains indications relating to the following items:

I	☒	Basis of the opinion
II	☐	Priority
III	☒	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain documents cited
VII	☐	Certain defects in the international application
VIII	☐	Certain observations on the international application

Date of submission of the demand	Date of completion of this report
16.03.2004	16.11.2004
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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/CA 03/01323

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

Description, Pages

1-87 as originally filed

Claims, Numbers

1-24 received on 25.10.2004 with letter of 21.10.2004

Drawings, Sheets

1/25-25/25 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☒ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)).

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

   see separate sheet

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application,

   ☒ claims Nos. 8-10,13-17

   because:

   ☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (specify):

   ☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. are so unclear that no meaningful opinion could be formed *(specify)*:

   ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

   ☒ no international search report has been established for the said claims Nos. 8-10,13-17

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the Standard.

   ☐ the computer readable form has not been furnished or does not comply with the Standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	5-7,11,18
	No:	Claims	1-4,12,18
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-7,11,12,18
Industrial applicability (IA)	Yes:	Claims	1-7,11,12,18
	No:	Claims	

2. Citations and explanations

see separate sheet

Re item I:

The amendments filed with the letter dated 21.10.2004 introduce subject-matter which extends beyond the content of the application as filed, contrary to Article 34(2)(b) PCT. The amendments concerned are the following:

a) Present claim 1 now refers to the identification of a genomic sequence from a first fish species containing a nucleotide sequence encoding the initial peptide as well as to screening a test nucleic acid sequence from a fish species other than the first fish species. The basis for these amendment indicated by the applicant, namely p. 15-16, 18, and 36-37 refer either to a method which departs from winter flounder pleurocidin-like sequences and which leads to the isolation of additional pleurcidin-like sequences from total RNA of a small number of specified fish species using primers which, contrary to the suggestion of present claim 1, are not flanking the initial peptide (PL5' and PL3'), or to a method of isolating additional hepcidin-like sequences from genomic DNA of a small number of specified fish species using primers specific for conserved sequences in the signal peptide of all reported hepcidins in combination with primers based on highly conserved sequences in the 3'UTR of salmon and flatfish. In view of these specific disclosures the method of present claim 1 is considered an undue generalisation which has no basis in the application as filed.

b) Present claim 3 refers to a cleavage product of histone 2A from catfish other than a parasin. This disclaimer has no basis in the application as filed.

c) The considerations of I a) also apply to present claim 20 and present claim 15, the latter now referring to present claim 1.

d) Present claims 20 and 23 refer to modifications of the peptides listed as well as to substitutions or deletions within said peptides for which no basis can be found in the application as filed. Moreover, the peptides according to claim 20(q) - (aw) do not have a basis in the application as originally filed.

e) The foregoing considerations also apply to the nucleic acids encoding the said

undisclosed peptides as falling under the scope of present claims 22 and 24.

As one of the independent claims under investigation, namely claim 1 relates to added matter, the Preliminary Examination Report was established on the basis of the set of claims as originally filed (R. 70.2(c) PCT).

Re item IV:

The International Preliminary Examination Authority (IPEA) agrees with the observation of the International Search Authority (ISA) as to lack of unity of invention and identifies the following inventions:

Invention 1: claims 1-7,11,12,18 (completely)

A method of identifying candidate nucleic acid sequences encoding antimicrobial peptides, a kit for performing said method, use as defined in claim 12.

Inventions 2-22: claims 8-10, 13-17 (all partially)

An isolated pleurocidin-like peptide comprising a sequence at least 80% homologous to either peptide a or b and represented by one of the sequences depicted in Table 4, an isolated nucleic acid sequence depicted in Appendix I encoding the said peptide.

Inventions 23-55: claims 8, 10, 16 (all partially)

An isolated nucleic acid sequence depicted in Appendix I and not encoding a peptide which falls under the definition of inventions 2-22

Inventions 56-94: claims 8-10, 13-17 (all partially)

An isolated hepcidin peptide comprising a sequence at least 80% homologous to any of peptides c-f and represented by one of the sequences depicted in Table 13, an isolated nucleic acid sequence depicted in Appendix II encoding

the said peptide .

The reasons for the lack of unity being as follows:

a) The only identifiable technical feature that all inventions have in common is that they refer to antimicrobial peptides and nucleic acids encoding them. Inventions 2-22 further share the technical feature of relating to pleurocidin-like peptides, whereas inventions 56-94 refer to hepcins.

b) The said features do not provide a unifying concept as they are known from the prior art. Douglas et al., Developmental and Comparative Immunology, 25:137-147 (A1) teaches pleurocidin-like peptides, nucleic acids encoding them as well as methods of identifying the latter in winter flounder. The pleurocidin-like peptides disclosed comprise a peptide 80% homologous to the peptides a and b according to claim 13. Moreover, A1 teaches the pleurocidin-like peptides indicated in Table 4 by the codes NRC-01, NRC-02, NRC-04, NRC-05 and NRC-05. Cole et al., JBC, 272:12008-12013 (A2) teaches the isolation of pleurocidin from winter flounder comprising a peptide 80% homologous to the peptides a and b according to claim 13. Cole et al., Antimicrobial Agents and Chemotherapy, 44:2039-2045 (A3) teaches the pleurocidin of winter flounder comprising a peptide 80% homologous to the peptides a and b according to claim 13 as well as a nucleic acid encoding the said pleurocidin and a method of identifying the said nucleic acid. US 6288212 (A4) discloses pleurocidin analogues comprising a peptide 80% homologous to the peptides a and b according to claim 13. Shike et al. Eur. J. Biochem., 269:2232-2237 (A5) teaches hepcidins from bass, winter flounder, rainbow trout and atlantic salmon which comprise a peptide 80% homologous to the peptides d and f according to claim 13 as well as a hepcidin of long-jawed mudsucker comprising a peptide 80% homologous to the peptides c and e according to claim 13. A5 furthermore teaches a nucleic acid encoding the hepcidin of bass as well as a method of identifying the said nucleic acid. Bayne et al., Developmental and Comparative Immunology, 25:205-217 (A6) teaches a hepcidin of rainbow trout comprising a peptide 80% homologous to the peptides d and f according to claim 13 as well as a nucleic acid encoding the said hepcidin and a method of identifying the said nucleic acid. Genbank Accession No. BI468191 (A7) discloses a nucleic acid encoding a hepcidin of atlantic

salmon. Genbank Accession No. AW013026 (A8) discloses a nucleic acid encoding a hepcidin of winter flounder. Genbank-Accession No. AW783824 (A9) discloses a nucleic acid encoding a hepcidin of long-jawed mudsucker.

c) In view of the prior art represented by A1-A9, the problems of the underlying application can be defined as i) the provision of a further method of identifying candidate nucleic acid sequences encoding antimicrobial peptides, ii) the provision of further pleurocidin-like peptides or hepcidins and/or of nucleic acids encoding them and iii) the provision of further nucleic acids possibly encoding further pleurocidin-like peptides.

d) Each of the inventions listed above represents an independent solution concerning one of the foregoing problems of the underlying application. Solution 1 is the provision of a method of identifying candidate nucleic acid sequences encoding antimicrobial peptides using primers flanking genes encoding known antimicrobial peptides. Each of the solutions 2-22 provides one of the pleurocidin-like peptides depicted in Table 4 as well as a nucleic acid encoding it as depicted in Appendix I. Each of the solutions 23-55 provides a nucleic acid as depicted in Appendix I, possibly encoding a pleuricidin-like peptide which is however different form the peptides given in Table 4. Each of solutions 56-94 provides one of the hepcidins depicted in Table 13 as well as a nucleic acid encoding it.

e) The pleurocidin-like sequences given in Table 4 appear to contain both consensus sequences (peptide a and b) given in claim 13. Also the hepcidins according to Table 13 appear to contain more than one of the consensus sequences (peptides c-f) of claim 13. Therefore, the said consensus sequences do not provide a basis for grouping the inventions. Moreover, pleurocidin-like peptides and hepcidin comprising the said consensus peptides are known from the prior art (supra), and thus these consensus sequences not provide a unifying concept for groups of the antimicrobial peptides according Tables 4 and/or 13.

f) The assignment of nucleic acids to their encoded antimicrobial peptides is based on the NRC codes given in Tables 4 and 13 as well as in Appendices I

and II. Since codes NRC-101 - NRC-133 of Appendix I do not match a peptide in Table 4, the corresponding nucleic acids were considered to be unrelated to pleurocidin-like peptides given in Table 4. Consequently, these nucleic acids were considered to provide independent solutions to the problem of providing further sequences of pleurocidin-like genes.

g) In view of the fact that methods of identifying antimicrobial peptides, as well as pleuricidin-like peptides and hepcidins comprising peptides at least 80% homologous to one of the peptides a-f of claim 13 as well as nucleic acids encoding them are already known from A-A9; due to the otherwise essential differences in primary structure of the different pleurocidin-like peptides and hepcidins as well as of the nucleic acids encoding them; and due to the fact that no other technical features can be distinguished which, in the light of the prior art could be regarded as special technical features common to the above solutions, the IPEA is of the opinion that there is no single inventive concept in the sense of R. 13.1 PCT underlying the 94 solutions contained in the present application. Consequently, there is a lack of unity, and different inventions have been formulated as different subjects as done above.

h) The ISA has searched the first invention (claims 1-7 and 18).

Re item V:

1. Reference is made to the following documents:

D1: Douglas, S.E. et al., Developmental and Comparative Immunology 25:137-147 (2001) cited in the present application.
D2: Douglas, S.E. et al., Developmental and Comparative Immunology 27:589-601 (07-2003)

2. D1 discloses the isolation of fragment of genomic DNA of winter flounder encoding further pleurocidin-like peptides (p. 138, c. 2, para. 2 -p. 142, c. 1, para. 1). The method of isolation starts from a known winter flounder pleurocidin peptide. Degenerated oligonucleotide probes are used to isolate the cDNA encoding said known pleurocidin from a winter flounder cDNA library. Then the said genomic DNA

fragments are isolated using primers which bind to sequences flanking the sequence encoding said known mature pleurocidin as determined from the said cDNA (Table 1). Furthermore, the term "genomic DNA" as used throughout the application also refers to cDNA. Hence, the subject-matter of claims 1, 12 and 18 lacks novelty over D1 (Art. 33(2) PCT).

3.1 The additional features suggested by claim 2 are inherent features of the pleurocidin used in D1 as a starting point for the isolation of further pleurocidin-like sequences. These features thus do not establish novelty (Art. 33(2) PCT).

3.2 The additional features suggested by claims 3 and 4 are also disclosed in D1 (supra) and do not establish novelty (Art. 33(2) PCT).

3.3 The subject-matter of claims 6, 7 and 11 is novel as none of the prior art documents discloses the combination of features suggested by said claims (Art. 33(2) PCT).

4.1 D1 discloses two pairs of flanking primers binding to sequences flanking sequences encoding a pleurocidin (Table 1) and the use thereof to isolate nucleic acids encoding further pleurocidin-like peptides (supra). Inclusion of said primers together with instructions for performing the method of D1 into a kit requires just routine skills. Hence, the subject-matter of claim 11 is not considered to involve an inventive step (Art. 33(3) PCT).

4.2 The additional features suggested by claims 5-7 refer to routinely applied experimentation in the field of the invention which do not result in any unforeseeable technical effect and thus do not establish an inventive step (Art. 33(3) PCT).

5. Industrial applicability of the subject-matter of claims 1-7, 11, 12 and 18 is acknowledged (Art. 33(4) PCT).

6.1 It is clear from the description that the following features are essential to the definition of the invention:

The flanking sequences targeted by the primers for amplification are conserved sequences and encode the pre and pro region of pleurocidin or the pre region and

the 3'UTR of hepcidin.

Since independent claims 1, 11 and 18 do not contain these features they do not meet the requirement following from Art. 6 PCT taken in combination with R. 6.3(b) PCT that any independent claim must contain all the technical features essential to the definition of the invention.

6.2 The method of claim 1 appears only supported insofar as it relates to the identification of pleurocidin or hepcidin encoding sequences. No support is provided for a method in which other antibacterial peptides, in particular those referred to in claim 3 are isolated (Art. 6 PCT). As outlined under V 6.1 above, the method of the invention is based on the observation that nucleic acids encoding hepcidins and pleurocidin-like peptides of different fish are flanked by conserved sequences which can be used to identify further hepcidins and pleurocidin-like peptides, respectively. For such a method to be applicable to the identification of further antimicrobial peptides in general and in particular for further peptides according to claim 3, sufficient experimental support would be required to demonstrate that sequences flanking sequences which encode antimicrobial peptides in fish are generally conserved. However, the description only supports such a sequence conservation for sequences encoding hepcidins and pleurocidin-like peptides.

6.3 In claim 3 the terms "parasin" and "cleavage product of histone 2A from catfish" are used to refer to two different antimicrobial peptides. However, parasin is a cleavage product of histone 2A from catfish (Art. 6 PCT).

6.4 Claim 11 refers to a kit which is considered as being a composition of entities. The claim comprises as an additional feature instructions for using the reagents contained in said kit. The only technical features such instructions possibly refer to are the steps characterising the methods they refer to. Hence, such instructions are considered to characterise a method of using a kit, rather than the kit per se, and as such obscure the scope of the claim since its category is no longer clear (Art. 6 PCT).

6.5 The terms used in claim 12 to refer to peptide regions have no well recognised technical meaning thereby rendering the scope of said claims unclear (Art. 6 PCT). These terms should have been replaced by the definition of said terms as given on p.

7, l. 14 - p. 9, l. 20).

7. Certain published documents (Rule 70.10)
Should the priority of the present application not be valid, the D2 would be relevant with respect to novelty and inventive step (Art. 33(2) and (3) PCT).

We Claim:

1. A method of identifying candidate nucleic acid sequences encoding antimicrobial peptides, said method comprising:

a) identifying an initial peptide of interest;

b) identifying genomic DNA encoding the initial peptide;

c) identifying a flanking sequence on each side of the initial peptide;

d) obtaining primers complementary to the flanking sequences; and,

e) screening a wide range of nucleic acid sequences to identify candidate sequences capable of being amplified using the primers from step d).

2. The method of claim 1 wherein the initial peptide of interest has a net positive charge of at least 2 and has an amphipathic structure.

3. The method of claim 1 wherein the initial peptide of interest is a hepcidin, a pleurocidin, a pardaxin, a misgurin, HFA-1, a piscidin, a moronecidin, a parasin, or a cleavage product of histone 2A from catfish.

4. The method of claim 1 wherein the initial peptide of interest is a hepcidin or a pleurocidin.

5. The method of claim 1, 2 or 3 comprising a further step g) of predicting the amino acid sequence encoded by the candidate sequence and selecting nucleic acid sequences which are predicted to encode peptides having an amphipathic structure and a net charge.

6. The method of claim 5 comprising a further additional steps of obtaining a peptide corresponding to the candidate nucleic acid sequence and assaying the peptide sequence for antimicrobial activity.

7. The method of claim 1 comprising a further step (a') of confirming that the initial peptide of interest has antimicrobial activity.

8. An isolated nucleic acid sequence identifiable using the method of any preceding claim.

9. An isolated polypeptide capable of being encoded by the nucleic acid sequence of claim 8.

10. An isolated nucleic acid sequence comprising a flanking sequence.

11. A kit comprising:

a. a first nucleic acid sequence at least 95 % identical to a first flanking sequence, located at or near a 5' end of a target sequence encoding an antimicrobial peptide;

b. a second nucleic acid sequence at least 95 % identical to a second flanking sequence located at or near a 3' end of a target sequence encoding an antimicrobial peptide; and

c. instructions for carrying out the method of claim 1.

12. Use of at least one of signal sequence I, acidic sequence I, signal peptide II, signal peptide III, signal peptide IV, signal peptide V, prosequence I, prosequence II, nucleic acid sequences encoding them, and nucleic acid sequences substantially complementary to such encoding nucleic acids, in the identification or amplification of antimicrobial peptides.

13. An isolated antimicrobial peptide at least 80% homologous to one of peptide a, b, c or d:

Peptide a	GW(G/K)XXFXK
Peptide b	GXXXXXXXHXGXXIH
Peptide c	FKCKFCCGCCXXGVCGXCC
Peptide d	CXXCCNCC(K/H)XKGCGFCCKF
Peptide e	FKCKFCCGCRCGXXCGLCCKF
Peptide f	XXXCXXCCNXXGCGXCCKX

14. The antimicrobial peptide of claim 13 which is at least 90% homologous to one of peptide a, b c or d.

15. The antimicrobial peptide of claim 13 which is one of peptide a, b, c or d.

16. An isolated nucleic acid sequence depicted in Appendix I or Appendix II.

17. An isolated nucleic acid sequence depicted in Table 4 or 13.

18. A method of identifying candidate nucleic acid sequences encoding antimicrobial peptides, said method comprising:

a) identifying a nucleic acid sequence encoding an initial peptide of interest;

b) identifying genomic DNA encoding the initial peptide;

c) identifying a flanking sequence on each side of the initial peptide;

d) obtaining primers complementary to the flanking sequences; and,

e) screening a wide range of nucleic acid sequences to identify candidate sequences capable of being amplified using the primers from step d).

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